Synthesis of Fluorescent Muramyl Dipeptide Congeners. 2

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Muramyl dipeptide (MDP) analogues were prepared and utilized in the synthesis of new fluorescently labeled MDP derivatives for use as biologic probes. Thus, N^{α}-(*N*-acetylmuramyl)-L-lysyl-D-isoglutamine (Lys-MDP, **4**) and N^{α}-(*N*-acetylmuramyl)-L-alanyl-D-isogutaminyl)-L-lysine [MTP, **5**] were synthesized and then reacted with 2-(fluoresceinylamino)-4,6-dichloro-*s*-triazine (DTAF, **2**) to yield the fluorescent adducts, DTAF-Lys-MDP (**6**) and DTAF-MTP (**7**). The adjuvant activity of the fluorescent MDP derivatives was determined by the ability of the compounds to promote delayed skin test responses in guinea pigs immunized with ovalbumin (OA) and by evaluating the anti-OA activity of these guinea pigs.

In a previous paper[1] we reported the preparation of a novel fluorescently labeled muramyl dipeptide as a potential tool for studying the role of MDP in immunity. *N*-(Acetylmuramyl)-L-alanyl-D-isoglutamine (MDP, **1**) was conjugated to dichloro-*s*-triazinoaminofluorescein (DTAF, **2**) to form the fluorescent adduct DTAF-MDP (**3**). DTAF-MDP possessed adjuvant activity both in vitro[1,2] and in vivo. Through the use of fluorescence microscopy and flow cytometry, **3** has been utilized for the study of the existence and prevalence of cell membrane binding sites that are specific for the MDP molecule.

Fluorescence microscopy has revealed that **3** labels rabbit lung macrophages, but two patterns of staining were observed: (1) uniform membrane fluorescence and (2) internal fluorescence in the presence of membrane fluorescence. Concern for the second staining pattern is based upon its occurrence even in the presence of sodium azide, which is used to inhibit endocytosis, the incorporation of substances into a cell phagocytosis or pinocytosis. The intensity of the internal fluorescence is greater than the membrane fluorescence, which makes data obtained by flow cytometry more difficult to interpret.

Although **3** exhibits positive adjuvant activity, there is no evidence that the labile ether bond between MDP and DTAF remains intact in biological systems. The detection of internalized fluorescent label within cells incubated with **3** may represent free DTAF obtained by cleavage of the ether linkage concomitant with the phagocytosis of the dye portion of the molecule. Even if **3** survives as an intact molecule, the bulky fluorescent dye moiety may cause it to exhibit different cell-binding specificity than the parent MDP molecule.

To examine the issues of molecular stability and cell-binding specificity and to study the role of **1** in immunity, we designed fluorescent MDP analogues in which the fluorescent label is incorporated on portions of the molecule different from the carbohydrate hydroxyls. It is necessary that the derivatives selected possess adjuvant potency similar to the parent compound and that each contains a functional group suitable for reacting with the fluorescent dye. Structure-activity relationship studies on **1**[3-7] reveal that a variety of amino acids can replace L-alanine with retention of adjuvant activity. It has been demonstrated that the γ-carboxyl function of D-isoglutamine can be coupled to various amino acids without loss of adjuvant activity.[8,9] The introduction of an amino acid on **1**, which contains a functionality suitable for coupling to **2**, would allow the preparation of novel fluorescent MDP compounds.

Studies by Kamisango et al.[3] indicated that the L-alanyl residue could be replaced by basic amino acids as long as the amine was amidated. This led us to use N^{α}-(*N*-acetylmuramyl)-L-lysyl-D-isoglutamine (Lys-MDP, **4**)[3] as the basis for the preparation of second fluorescent analogue. Audibert and co-workers[8] have shown that lysine can be coupled through the γ-carboxyl of the D-isoglutamine residue of **1** without loss of adjuvant activity. On the basis of these findings, we selected N^{α}-(*N*-acetylmuramyl)-L-alanyl-D-isoglutaminyl-L-lysine (MTP, **5**)[8] to serve as the basis for our third fluorescent analogue. The amino groups in these compounds were desirable for selective reactions with the fluorescent dye, DTAF, since **2** reacts much faster with *n*-alkyl amines than with primary alcohols.[10,11] The long *n*-alkyl side chains would allow the attachment of the dye through an amino bond, which is more stable than the labile ether bond of **3**, and the long side chain would provide a spacer between the adjuvant portion and the fluorescent dye, which may lessen the steric strain and hinderance.

We report that Lys-MDP (**4**) and MTP (**5**) react efficently with **2** to yield the fluorescent MDP derivatives DTAF-Lys-MDP (**6**) and DTAF-MTP (**7**), respectively (Figure 2). Compounds **6** and **7** possess interesting biological activities and exhibit different cell-binding specificities.

Chemistry

The synthetic route from the synthesis of **4** and **5** is analogous to the route used to prepare **1**.[1] N^{α}-BOC-N^{ϵ}-Z-L-lysine (**8**),[12] was coupled to the perchlorate salt of

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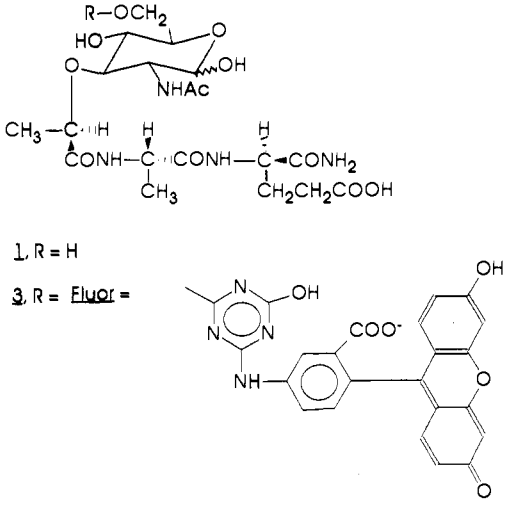
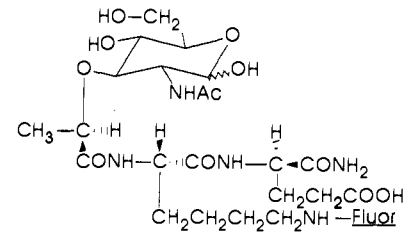


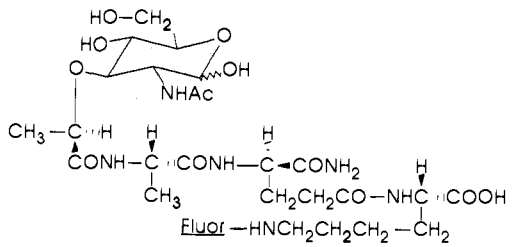
1, R = H

3, R = Fluor =

Figure 1.



6, DTAF Lys MDP



7, DTAF MTP

Figure 2.

benzyl D-isoglutaminate (**9**), by using the mixed anhydride coupling method,[13] to give the protected dipeptide **10**. Trifluoroacetic acid (TFA) was used to selectively remove the BOC group from the dipeptide without cleaving the Z protecting group. Compound **11** was coupled to benzyl *N*-acetyl-4,6-*O*-benzylidene muramic acid (**12**) to produce the fully protected Lys-MDP (**13**); **13** was treated with hot acetic acid and then hydrogenated over palladium on charcoal to give **4**. High-resolution proton NMR spectra, fast atom bombardment mass spectra (FABMS), and specific rotation measurements verified that the product (**4**) was Lys-MDP.

MTP (**5**) was prepared by shorter route from the protected MDP (**14**). Only the benzyl ester of **14** is hydrogen-labile in a nonacidic environment and can be cleaved in a matter of minutes when methanol is the solvent. The resulting product (**15**) was coupled with benzyl-N^{ϵ}-*Z*-L-lysine tosylate (**16**)[14] to give the fully protected MTP (**17**). Treatment of **17** with hot acetic acid followed by hydrogenation over palladium on charchoal gave the desired MTP (**5**). High-resolution proton NMR, FABMS, and specific rotation measurements verified that **5** was obtained.

Table I. Adjuvant Activity of MDP Derivatives in Guinea Pigs

treatment[a]	DTH[b]	antibody response[c]
control	0	56.97 ± 22.0
MDP (1)	10.6 ± 2.0	82.49 ± 15.6
DTAF-MDP (3)	11.5 ± 2.0	123.34 ± 23.5
DTAF-MTP (7)	20.2 ± 1.2	192.37 ± 41.1
DTAF-Lys-MDP (6)	2.9 ± 1.7	17.65 ± 5.7

[a] Guinea pigs received in each posterior foot pad 0.1 mL of saline in incomplete Freund's adjuvant containing 0.5 mg of OA with or without MDP or a derivative. [b] DTH quantitated by measuring the diameter of the induration skin test reaction 48 h after intradermal injection of 10 μg of OA in saline. [c] ELISA activity compared to a reference standard of 100 units obtained by pooling the individual sera. Data presented as group mean ± 1 SEM.

The fluorescently labeled MDP analogues **6** and **7** were prepared by reacting the free amino moieties on **4** and **5** with DTAF (**2**) in an aqueous potassium carbonate (pH 10) solution at room temperature. The reactions were concentrated, and the crude products were eluted twice on Sephadex G-15 filtration columns.

The fastest moving bands were collected and lyophilized to give **6** and **7**. Low-resolution proton NMR suggests that a 1:1 ratio exists between the fluorescent label and the respective MDP analogue. High-resolution proton NMR shows chemical shift changes in the dipeptide region of the spectra, indicative that the dye (**2**) is coupled in each case to the lysyl residue.

Biological Results and Discussion

Adjuvant activity of the MDP analogues was determined by the ability of the compounds to stimulate delayed hypersensitivity to ovalbumin (OA) in guinea pigs and to boost the anti-OA activity of these guinea pigs.[15] Results of the skin test and antibody assays are presented in Table I.

The results of the skin testing demonstrate that the three fluorescently labeled MDP analogues (**3**, **6**, and **7**) differ in their ability to sensitize guinea pigs for delayed hypersensitivity. The diameter of the OA skin test reactions obtained in guinea pigs immunized with OA plus **3** was essentially identical with that produced by OA plus 1 control group, although the test sites of 3/4 animals in this group had less induration. In contrast, OA plus **7** immunized guinea pigs had exaggerated skin test responses to OA with 2/4 animals having central necrosis at the OA skin test site; **6** appeared to have minimal adjuvant activity, with 2/4 animals showing no skin test response to OA and the others having a small zone of erythema with minimal induration.

The results of the antibody studies must be interpreted with some caution since 3 days elapsed between skin testing and sacrifice, a time sufficient for a minimal boosting effect. However, a pattern similar to that of the skin testing was obtained with the anti-OA responses to the various fluorescently labeled MDP analogues. Compound **3** produced an anti-OA response similar to the MDP (**1**) control, while **7** produced an exaggerated antibody response. Animals immunized with **6** produced comparatively low levels of anti-OA antibodies.

Experimental Section

Low-resolution (LR) proton nuclear magnetic resonance (NMR) spectra were generated on a Varian Associates EM 360-MHz ^{1}H NMR instrument. High-resolution (HR) proton NMR spectra

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were obtained on a Bruker WM 360-MHz variable probe NMR instrument. All NMR spectra were obtained with use of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as the internal standard. Elemental microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, TN. Sephadex G-15 (Sigma Chemical Co.) was used for column chromatography.

N^{α}-(*N*-Acetylmuramyl)-N^{ϵ}-DTAF-L-lysyl-D-isoglutamine (DTAF-Lys-MDP, 6). Lys-MDP (4) (55.2 mg, 0.10 mmol) and DTAF (2) (65.1 mg, 0.12 mmol) were added to 10 mL of a 0.25 M K_2CO_3 solution, and the reaction was stirred overnight. Amine detection on TLC ($BuOH/AcOH/H_2O$, 4:1:4; top layer) with ninhydrin showed no detectable unreacted Lys-MDP. The reaction was lyophilized to a solid and reconstituted in 2 mL of H_2O. The concentrate was eluted on a Sephadex G-15 gel filtration column (bed volume of 1.0 mg of dye to 2 mL of gel). The fastest moving band was collected, concentrated, and eluted on a second Sephadex G-15 column. Collection of the fastest moving band afforded the homogeneous fluorescent adduct DTAF-Lys-MDP (6): yield 61.8 mg (60%); NMR-LR (D_2O), integration ratio for the aromatic region (δ 6.0–8.5) to the peptide region (δ 1.0–3.0) was 1:2 (9 H to 16 h), which is consistent with a 1:1 ratio of DTAF to Lys-MDP; NMR-HR (D_2O) δ 1.36 (d, 3 H, Mur-CH_3), 1.38 (m, 2 H, $CH_2CH_2CH_2NH$), 1.97 (s, 3 H, $COCH_3$), 3.08 (m, 2 H, CH_2CH_2NH). Anal. ($C_{45}H_{49}N_9O_{16}ClK\cdot KHCO_3$) C, H, N.

***N*-(*N*-Acetylmuramyl)-L-alanyl-D-isoglutaminyl-*N*-DTAF-L-lysine (DTAF-MTP) (7).** MTP (5) (53.1 mg; 0.10 mmol) and DTAF (2) (68.2 mg, 0.13 mmol) were reacted overnight in 10 mL of 0.25 M K_2CO_3. The reaction workup was similar to the one used to purify 6. Elution of the reaction on Sephadex G 15 gave DTAF-MTP: yield 68.4 mg (61%); NMR-LR (D_2O), integration ratio for the aromatic region (δ 6.0–8.0) to the peptide region (δ 1.0–3.0) was 1:2 (9 H to 19 H), which is consistent with a 1:1 ratio of DTAF to MTP; NMR-HR (D_2O) δ 1.35 (d, 3 H, Mur-CH_3), 1.40 (d, 3 H, Ala-CH_3), 1.75 (m, 2 H, CH_2CH_2NH), 1.96 (s, 3 H, $COCH_3$), 3.07 (m, 2 H, CH_2NH). Anal. ($C_{48}H_{54}N_{10}O_{17}$,ClK·H_2O) C, H, N.

Biological Methods. Skin Testing. The immunization protocol used was previously described by Audibert et al.[15] Hartley strain guinea pigs, 400–600 g females, were obtained from CAMM and divided into groups of four animals. Each posteror footpad received an injection of 0.1 mL of a saline-incomplete Freund's adjuvant emulsion containing 0.5 mg of OA with or without MDP or its derivatives. The test group had MDP added to the emulsion at 50 μg/0.1 mL, and the various derivatives were adjusted to be at equimolar concentration to MDP. Guinea pigs were skin tested on day 14 with 0.1 mL of a saline solution containing 10 μg of OA. The skin-test response was determined by measuring the diameters of induration 48 h after intradermal injection of antigen.

Antibody Response. Guinea pigs were sacrificed by ip injection with pentobarbital 72 h after skin testing, and blood was obtained from the heart for serum antibody studies. Anti-OA activity was measured by an ELISA detection system,[16] and the activity was compared to that obtained by pooling the sera from all the guinea pigs and assigning the pool a value of 100 units/mL of activity. The lowest dilution of pooled serum that reacted in the assay was 1:265 000.

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The in Vitro Dental Plaque Inhibitory Properties of a Series of *N*-[1-Alkyl-4(1*H*)-pyridinylidene]alkylamines

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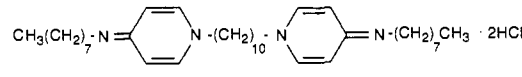
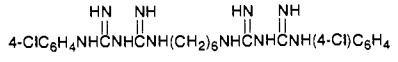
A series of novel *N*-[1-alkyl-4(1*H*)-pyridinylidene]alkylamine hydrohalides has been prepared and evaluated as inhibitors of dental plaque formation, in vitro. Several members of the series exhibited potency ca. 9-fold greater than that of chlorhexidine vs *Streptococcus sobrinus* 6715-13. The di-*n*-octyl analogue, 11 (pirtenidine), was found to be highly efficacious against several other oral plaque-forming microorganisms and is presently undergoing preclinical evaluation.

We have previously described a class of bispyridinamine antimicrobial agents that were inhibitors of dental plaque growth in vitro.[1] From this series emerged a compound, octenidine, which demonstrated clinical efficacy in inhibiting human dental plaque formation[2,3] and which has been shown in animal models to reduce caries formation[4] and improve gingival health.[5] We now report on a series of *N*-[1-alkyl-4(1*H*)-pyridinylidene]alkylamines (A) related to octenidine that have improved in vitro potency over the latter in laboratory models for inhibition of dental plaque.

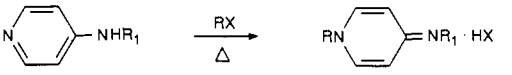


OCTENIDINE



CHLORHEXIDINE

Scheme I



A

Periodontal disease is the leading cause of tooth loss in man,[6] and dental plaque, the dense adhesive microbial mass that colonizes tooth surfaces, is strongly implicated

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